(*NEW SERIES*) **No. 42.**

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BY

OFFICERS OF THE MEDICAL AND SANITARY DEPARTMENTS

OF THE

GOVERNMENT OF INDIA.

PART I.

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BY

MAJOR E. R. ROST, I.M.S.

PART II.

The Cultivation of the Leprosy Bacillus

BY

CAPTAIN T. S. B. WILLIAMS, M.B., I.M.S.
(EDITED BY THE DIRECTOR-GENERAL, INDIAN MEDICAL SERVICE.)

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INTRODUCTION.

In 1904 Captain Rost announced that he had succeeded in cultivating the *Bacillus Lepræ* and in preparing from it a curative vaccine; but subsequent investigation went to prove that the so-called culture was really a contamination. When, therefore, in October 1909 it came to the notice of the Government of India that he again claimed to have obtained pure cultures of the bacillus, I determined to go to Rangoon and investigate his claims personally.

Careful examination of his cultures very soon convinced me that, whatever might have been the case in 1904, we were no longer dealing merely with a contamination, and that he had succeeded in isolating and cultivating an organism which possessed very distinctive characteristics.

About the same time, Captain Beauchamp Williams, who had been deputed to study the effects of Deycke's "Nastin" at the Matunga Leper Asylum, informed me that he had succeeded in growing from cases of leprosy, an organism very similar to Rost's. I therefore instructed Major Rost to place himself in communication with Captain Williams, and I obtained the permission of the Surgeon-General with the Government of Bombay for general supervision of their work by Lieutenant-Colonel Bannerman, Director of the Bombay Bacteriological Laboratory, whose covering letter I give below.

The results obtained by these officers cannot be regarded as being absolutely conclusive, but they are, in my opinion, of sufficient importance to justify their publication.

C. P. LUKIS, M.D., F.R.C.S.,
Director-General,
Indian Medical Service.

(Copy of letter from Lieut.-Colonel Bannerman, dated 21st September 1910.)

In accordance with instructions contained in your demi-official letter of 4th July 1910, I have the honour to report that I asked Major E. R. Rost, I.M.S., and Captain Beauchamp Williams, I.M.S., to compile a report of the work on leprosy in which they have respectively been engaged. These reports have now been furnished to me, and I beg to submit the following remarks with regard to them.

2. Before doing so, however, I would invite attention to a series of papers published in the "Lancet" of 26th February and 5th and 19th March of this year on the subject of "The Streptotrichoses and Tuberculosis." These form the Milroy lectures delivered before the Royal College of Physicians of London this year by Alexander G. R. Foulerton, F.R.C.S. The publication of these lectures occurred at a very opportune moment, for they focus in a convenient manner our present knowledge of the streptotriceæ.

From these lectures we learn that in all probability Koch's tubercle "bacillus" is not a bacillus at all, but a streptothrix and that many other diseases having an affinity to tuberculosis are certainly caused by streptotriceæ.

In the first Milroy lecture it is laid down that the streptotriceæ belong to a higher group of organisms than the bacilli (*fission fungi*) and should be included with the hyphomycetes or mould fungi. Their life-history is as follows:—The starting point is a spore, which, however, differs from an ordinary bacillary spore in staining reactions and in less resistance to heat. From this spore two or three threads sprout. "These threads gradually elongate so that at an early stage of growth one has a typical "ray fungus" consisting of three mycelial threads, usually of unequal length, radiating out from the position occupied by the spore as a centre. From these primary threads lateral branches in turn sprout out; and soon there is a densely tangled mass of mycelium representing the fully developed stage of the organism." The next stage is segmentation of these mycelial threads produced by transverse divisions and giving the appearance of a strepto-bacillary chain. The chain then breaks up into separate parts each of which has all the appearance of an ordinary bacillus, except that occasionally "a rod with the remains of a lateral branch may be seen." Along with this, or soon after, "a special kind of spore formation, chain sporulation may be observed" which when complete produces the appearance of "a streptococcal chain."

Variations in the above may be seen in individual species with regard to rapidity of growth, breadth of transverse divisions between the rods and frequency of lateral branching. When there is infrequent branching, the mycelial tuft is not easily distinguished from a mass of strepto-bacilli. "Indeed with some pathogenic species identification may be a difficult matter. In some cases typical ray fungi are not to be found either in pus from the lesions or in the tissues examined. In such cases all that one may find on microscopic examination is a number of rod segments which stain, often in a somewhat granular fashion, by Gram's method, and are occasionally acid-fast." This last, be it noted, is of special importance with regard to the organism from these lepers.

On attempting to cultivate one often finds the growth to be very slow and scanty on solid media, the result being "small, whitish, dry-looking, heaped-up colonies after six or seven days' incubation or not until after a longer period."

In peptone broth "after a few days' incubation small speck-like colonies appear, either cohering in a filmy mass at the bottom of the medium or adhering, isolated, to the sides of the tube."

The spore formation of mould-fungi differs from that of bacilli or yeast in the following respects. The bacilli produce single endogenous spores, one bacillus producing one spore.

The yeasts produce multiple endogenous spores, two, three or four.

The mould-fungi possess special spore-bearing organs of various types, and do not produce spores in every part of the organism. Further, these spores of the mould-fungi do not stain by Moeller's method, as bacillary spores do, but, on the contrary, they stain deeply by Gram's method. They are much more easily affected by a high temperature than bacillary spores, a rise of 15°C. above the thermal death-point of the mycelial form being sufficient to kill them.

It is probable, Foulerton says, that in the parasitic condition of life, the development of spherical spores may not take place, but that the bacillary bodies into which the mycelium breaks up under these conditions, may act like spores as the transporters of the disease from one part of the body to another. This would account for the localised manifestation in many cases of tubercular leprosy and the sudden exacerbations that take place accompanied by fever. It is believed that these bacillary bodies are "probably spore-bodies of some kind" and capable of reproducing themselves directly.

Additional biological characteristics of the streptotriceæ are, pigment-formation, efflorescence on the surface of solid media, and a heaped-up appearance. Many develop acid-fast properties. In peptone broth they often grow in filmy masses at the bottom of the tubes.

3. Having obtained a general view of the life-history of the streptotriceæ, we may now turn to the organisms isolated by Major Rost and Captain Williams. In accordance with your instructions Major Rost supplied me with cultures of the organism separated by him from the nodules of lepers in Rangoon. These have been carefully examined in this Laboratory by Captain Williams and myself. Further, all the work done by Captain Williams in the cultivation of an organism from lepers at Matunga, has been done in my room, under my direct supervision. I am, therefore, in a position to discuss the reports submitted by Major Rost and Captain Williams in the light of the above experience. It appears certain that by using Major Rost's salt-free medium it is possible to obtain a growth from a certain proportion of

leper nodules. Both in our results obtained here and also in those obtained by Major Rost in Rangoon, the growth appears in about three days, at the bottom of the tube. Smears made from the growth at the bottom of the tube show acid-fast bacilli, similar morphologically to those seen in smears made from leprous nodules. Whether these are the actual bacilli of leprosy or not, it is a noteworthy fact that no growth is obtained under similar conditions in the controls on ordinary media. The organism is highly pleomorphic and likewise varies in its staining reactions, all stages between complete acid-fastness and the reverse being seen in a single preparation. These variations in form and staining reaction are quite compatible with the life-history of the streptotriceæ as given by Foulerton, and with its being a pure cultivation. The above remarks have reference especially to the bacillary form of growth as isolated in Rangoon and here. As will be seen by a reference to Williams' paper a very definite streptothrix has been grown by him in ordinary media from two cases in Persia and Bombay. In our examination of Rost's organism, we also convinced ourselves that one species at least produced at one stage a definite mycelial growth and even those, where a mycelial stage has not been seen, are certainly streptotricieæ. Further Deycke has grown a streptothrix from cases of leprosy which is very similar to that produced by Williams. A consideration of all these points together with the evidence produced in Williams' paper as to the streptothrix and bacillary form being but phases of the same organism, gives one ground for seriously considering anew the whole question of leprosy, and it seems to me that there is now a considerable amount of weight to be attached to the view that leprosy is very probably after all a streptothrix disease.

4. From a study of the papers of Rost and Williams and from a knowledge of the work done in this Laboratory, I am of opinion that the organisms found by Rost and Williams are probably identical. It will be seen from a perusal of their papers that in Rost's special medium their results are similar and the ultimate result has been the production of acid-fast organisms by both which are similar in their pleomorphism, acid-fastness, being streptotriceæ, and in their power to produce marked reaction in lepers. The only difference at present is that Williams' growth is sticky, while Rost's growth is dry. From Major Rost's paper, it would seem that his growth was originally sticky, but has altered by prolonged sub-culture.* It is also very interesting to note that Williams has grown several times a streptothrix which is very like that of Deycke, as recorded in his published results, and its growth on milk is

* Since the above was written, Captain Williams has been to Rangoon and has demonstrated that, under certain conditions, Major Rost's organism loses its wrinkled character and becomes sticky; whilst the organism isolated by himself is capable of growing as a wrinkled membrane.—(EDITOR)

strikingly similar to the growth of Professor Deycke's organism. Williams is of opinion, and I am somewhat inclined to agree with him, that his work links up that of Deycke and Major Rost, and that his two organisms are, really, one and the same organism, which has been very decidedly altered by its environment, and by the original medium in which it was grown. The details given in Williams' paper, and the fact that both his organisms produce an apparently specific reaction in lepers, such as is produced by Deycke's and Rost's organisms, would seem to be strong evidence in favour of this view.

5. In conclusion, I would like shortly to discuss the possibilities of successful treatment of lepers by a vaccine from these organisms. Major Rost describes in his paper very satisfactory results, some of which, I understand, you have yourself seen when you were in Rangoon. We have here for the last few months been using a vaccine made first of all from the streptothrix grown on milk, and later from the bacillary form of the organism grown from Rost's medium. Our results are most encouraging and, in our opinion, tend to confirm Major Rost's work.

Part I.

THE CULTIVATION OF THE BACILLUS OF LEPROSY AND THE TREATMENT OF CASES BY MEANS OF A VACCINE PREPARED FROM THE CULTIVATIONS.

BY

MAJOR E. R. ROST, I.M.S.

AFTER research into the Bacteriology of Leprosy extending over a period of seven years, I succeeded in 1909, with the assistance of my Clinical Assistant, Mr. Bansi Lal, in isolating from three cases of Leprosy an acid-fast bacillus, which I was able to subculture in pure growths through successive generations, and which possessed certain peculiar characteristics, resemblnig morphologically the bacillus of Leprosy.

The first case from which I succeeded in isolating the bacillus was one of long standing nodular Leprosy in a Chinaman, who periodically developed large blisters, which appeared spontaneously on anæsthetic areas without being caused by burns or irritants. Microscopic examination of the blister fluid showed in some instances a few acid-fast bacteria. The blister fluid drawn off into pipettes and incubated at room temperature for five days showed a marked increase in the number of acid-fast bacteria, and controls taken from the pipettes in ordinary media remained sterile. The pipettes showed white flakes, and, on microscopic examination, acid-fast bacteria were found massed together in parallel arrangement.

The medium used consisted of 250 c.c. of distilled volatile alkaloid of rotten fish, 250 c.c. of weak Lemco broth without salt or peptone and 50 c.c. of milk and was prepared in the following way :—

A bottle containing (Ngapee)* Burmese preserved fish is provided with a rubber stopper perforated with 2 holes through which steam could enter down a glass tube to the bottom of the bottle and pass over the contents and issue through a glass tube at the top; this bottle is placed in an autoclave and the outlet tube from the bottle led out through the autoclave into a condenser.

* Major Rost, perhaps, attaches too much importance to the Ngapee distillate. Williams has succeeded in growing the Leprosy bacillus in a medium in which distilled water is substituted for the Ngapee distillate.—Ed.

Steam generated in the autoclave passes over the heated fish (which has a little caustic soda added in order to bring out the volatile alkaloids more readily) and passes out of the autoclave into the condenser and is collected in a flask.

The medium is then mixed with previously prepared and autoclaved Lemco broth and milk in the above proportions, placed in tubes and autoclaved.

The medium was controlled on ordinary agar and broth at the time of the experiments. Tubes of this medium were inoculated from the blister fluid which had been previously left in pipettes at room temperature for five days. These pipettes now contained large masses of acid-fast bacteria, resembling morphologically that of Leprosy, whereas previously only a few were found in the blister fluid.

Three days later a slight stringy growth was visible at the bottom of the tube and films showed a fairly large number of acid-fast bacteria, massed together in parallel arrangement.

It was then found that a feebly acid-fast bacillus grew on nutrient agar and broth (without salt or peptone) in subcultures, and that the acid-fastness of the bacillus was regained by growing in milk, and that the degree of acid-fastness could be varied according to the fatty nature of the medium. Plating experiments were then carried out and in these five loopfuls of the culture were stirred in the nutrient agar (without salt or peptone) cooled down to 40°C. Three loopfuls were taken from this tube and removed to a second tube of agar medium which was poured into a Petri dish. Three days later the plates showed discrete colonies about the size of a pin's head, opaque, orange-red, raised with the centre, humped and moist to the naked eye.

Examined with a lens these colonies were circular, opaque, warm orange-red coloured, with regular unbroken margins. One of these colonies was inoculated into a tube of the primary medium and this formed the basis of experiments with this first cultivation.

By continuing the sub-culture and by occasional plating a slight variation was obtained ; two slightly differing cultures being secured, which by successive subculture again became identical in appearance.

The second successful isolation, producing a bacillus the cultural characteristics of which are practically identical with the first and differ very slightly only in rate of growth and moisture of colonies, was obtained from a European* suffering from nodular Leprosy. The fluid exudation from a cut nodule was used in this case, inoculation into the special medium being direct. Incubation at room temperature 30°C. in this case took longer than in the former case, a visible growth appearing after three weeks.

* This case is now practically cured by the use of a vaccine prepared from these cultivations.

This appeared as a slightly yellowish red growth on the surface of the medium. Subculture on ordinary media without fat showed the same loss of acid-fastness, which could be varied according to the fatty nature of the medium.

This culture appeared to grow more slowly and to be slightly more sticky than the first isolation. It has gradually altered in characteristics, and, after 18 months of successive subculture has become more like the culture first isolated.

In two other cases the same organism was isolated ; but the cultures being found on plating to be impure, these cultivations were discontinued.

In November 1909, by adopting a medium similar to that used with the first pure culture, a growth was obtained from a nodular case of Leprosy. This was a Burman lad, aged 18, Kone Sein, who showed on his phalanges and face, a number of hard shotty nodules not larger than a pea, pyramidal shaped, glazed and shining. The method employed was to reflect the skin with aseptic precautions, over one of these recent nodules and squeeze out the fluid ; inoculate it into the special medium and control also in ordinary media.

The tubes were incubated at 30°C. The controls remained sterile.

The special media tubes showed a number of acid-fast clumps similar to those found in the leper juice after fourteen days. Later on, these acid-fast masses began to diminish and after the third week required a prolonged search to find them. During the fourth week a steady increase was observed in these acid-fast clumps and several acid-fast bacilli were found to occur singly as though the organism was multiplying, but it was only at the end of the fourth week that the whole culture medium was observed to be impregnated with acid-fast bacteria.

After the fifth week a thin pellicle was found on the surface, this consisted of small, greyish, coarse granular flakes of a crumbling consistence.

From the original culture, plates were inoculated and in these the colonies appeared after a week and showed similar characteristics to those previously isolated, with the exception that the rate of growth was in the first instance slower, the colour a lighter yellow and the acid-fastness constant whether grown on fatty media or not : otherwise the subcultures from this case, in appearance and peculiarities of growth as detailed below, are identical with the other previously isolated cultures.

First and second successful Isolation O. L. O. and T. L. O.—H. O. and H. T.	Third successful Isolation. Kone Sein.
1. Grows best on pure milk ; on milk 50, Lemco-broth 250 (without salt or peptone) and distilled rotten fish medium 250.	.....
2. Primary isolation growth has not been obtained by any other medium yet discovered.	.....

First and second successful Isolation O. L. O. and T. L. O.—H. O. and H. T.	Third successful Isolation Kone Sein.
3. It grows on primary cultivation slowly	.....
4. Can be subcultured direct from primary growth on to the other media.	Does not grow on first subculture in ordinary media but does so after successive subculture in special medium.
5. On all other media, except fish broth, it appears to degenerate rapidly into attenuated forms of bacilli and becoming feebly acid-fast, strong broth and peptone and salt enhancing this degeneration.	.....
6. It grows on pure starch and potato-glycerine but not on pure albumen.	.....
7. Its acid-fastness varies with the fatty nature of the media used: on milk it is acid-fast and on fish broth very much so.	Acid-fastness constant on all media.
	33% HNO_3 —2 mins.
	25% H_2SO_4 —$\frac{1}{2}$ hour.
Milk. Fish broth.	
25% H_2SO_4 . . . . 5 mins ± —2½ mins.	
12½% H_2SO_4 . . . . 20 „ ± —10 „	
5% H_2SO_4 . . . ½ hour ± —½ hour	
8. It stains readily in the cold with simple aqueous aniline dyes and is gram-positive.	.....
9. It is non-motile.	.....
10. Grows best at 30°C. After a few generations from the original culture it grows in 48 hours.	.....
11. It is exceedingly pleomorphic, appearing in different forms at different periods of its growth. After 48 hours' growth its appearance is as in the nodules of a Leper. Later on, or in unfavourable media and temperature, degenerate forms are found which double or treble their usual length with a monilliform arrangement and lose their acid-fastness. These break down after a few days into clumps of small acid-fast coccoid forms. The coccoid forms are more evident in some cultures than in others.	In Kone Sein culture the thread-like forms have not been found to occur.
I. First successful Isolation. II. Second successful Isolation.	III. Third successful Isolation.
12. In old cultures an orange-red colour is seen at the bottom of the tubes. In milk it grows first as a layer just under the cream. In broth it grows first at the bottom as a thin whitish loose deposit rendered visible by shaking the tube. The upper portion of the broth remaining clear for the first two days and then pinkish flakes forming on the surface which show the disintegrating forms.	It grows luxuriantly on pure milk as a layer under the cream, but the orange-red colour is absent. In old cultures the colour has a tinge of yellow but has not become red as the two previous isolations. On agar its growth is slower and it assumes a more crinkled appearance. On glycerinated agar the growth is more luxuriant. In broth it falls as a powdery deposit at the bottom of the tubes while the medium is not clouded, in glycerinated broth there is no superficial growth except after prolonged cultivation.

13. Films are best prepared by filtering a culture through filter paper ; thus removing fatty masses and better specimens are thus obtained.

14. The characters of the cultures have slightly changed over a period of eighteen months, the different isolations from the three different cases tending to become more exactly alike, except the Kone Sein culture which does not vary its acid-fastness.

15. The following test media showed in each case:—

	Growth.	Acid.	Gas.	Remarks.
Glucose Litmus . .	+	—	—	Colour of litmus taken up by the growth after some time, leaving clear fluid, but colour of litmus not changed.
Mannite „ . .	+	—	—	
Dulcite „ . .	+	—	—	
Lactose „ . .	+	—	—	
Bile Salt „ . .	—	—	—	

16. After exposure at 80°C. for 15 minutes no subculture was obtained.

17. 1 in 500 bile salt prevents growth in all cases, sheep's bile 1 per cent. prevents growth.

18. The indol reaction is negative.

It will be seen from the above tables that the only differences between the two first isolated cultures and the third or Kone Sein culture are colour, rate of first growth, extent of acid-fastness and the absence of long thread-like forms in the Kone Sein cultures. Otherwise the characteristics of the three are the same, their pleomorphism being so marked and peculiar as to lead one to imagine that the culture is not pure; the varying acid-fastness in the first two isolated cultures tending, on first examination, to lead one still further astray.

Inoculation Experiments.—Injections of culture alone and mixed with butter were made into guinea-pigs, white rats, and rabbits, subcutaneously, by tattooing, by feeding and intra-peritoneally without result.

A monkey repeatedly injected with culture, developed clinical signs of the disease and nodules appeared in which were found typical lepra bacilli, but I failed to produce a pure cultivation. The history of this case is as follows :—

This monkey was first injected intra-peritoneally in October 1909, the injections being continued weekly, and the method of inoculation being varied between intra-peritoneal injections, tattooing and applications of poultices of culture and subcutaneous inoculations.

In December 1909, he began to develop exacerbations of temperature, which varied between 100 and 103, and nodules were noticed over the superciliary ridges ; on the cheeks and inner aspect of the left ankle ; and on the dorsum of the left foot.

At the end of December, the hard mass on the ankle was found to contain pus and this, when examined under the microscope, contained several clumps of acid-fast bacteria, resembling in appearance Lepra bacilli.

Acid-fast bacilli in parallel arrangement were found situated inside cells in smears prepared from the pus.

The injections were then stopped.

In February 1910, the nodules on the superciliary ridges were examined microscopically, the monkey being chloroformed and a nodule being excised partly for the purpose of obtaining a cultivation and partly for microscopic examination. The microscopic smears from this nodule showed numerous masses of acid-fast bacillary groups in parallel arrangements, resembling exactly specimens obtained from the human subject.

Cultivation gave negative results.

The monkey being a small animal the nodular lesions were of course small and photography failed to reproduce what was more easily visible to the naked eye. The superciliary ridges were raised and thickened and had a glistening red appearance. The nodules on the cheeks appeared to subside in February as did the nodules on the superciliary ridge, but the fever continued, the monkey gradually wasting away. Examination of the blood showed nothing.

In April 1910, the monkey died apparently from inanition, and post-mortem examination revealed no abnormality of the internal organs.

Smears taken from the sub-nodular area of the eye-brows showed some small groups of acid-fast bacteria in parallel arrangement resembling Lepra bacilli.

Cultivation from these failed, owing to contamination by skin bacteria, although great care was taken to sterilize the superficial skin.

Eight other monkeys have been injected with cultures of varying days' growth, but so far no clinical sign of the disease has been observed.

The Preparation of a Vaccine.—The first vaccine used was prepared from the subcultures obtained after plating from the first primary isolation.

Bacteria removed from an agar slope culture were shaken up with distilled water and centrifugalized, the fluid being poured off and fresh distilled water added, and the deposit shaken up again, and again centrifugalized several times, so as to wash the culture and remove all external toxins. The deposit of bacteria, after final washing and centrifugalising, was dried and weighed and macerated with 7 per cent. glycerine and distilled water to make up a percentage solution. It was then placed in tubes and autoclaved, the tubes being sealed.

The sealed tubes were then placed on a shaking machine for a period extending over several weeks. Ten minims of 1 in 400 of this vaccine produced a slight febrile reaction in cases of leprosy, and its therapeutic usefulness has been very marked.

In May 1910 the vaccine was prepared from the Kone Sein culture, and since this date this culture has been used in the preparation of the vaccine.

Several modifications of preparation of this vaccine were tried previous to this, but the above mentioned method appeared to produce a vaccine having a greater clinical value.

Lately, the fatty substance of the bacteria has been extracted by shaking in ether over a period of several weeks, occasionally drawing off the ether and adding fresh, filtering and centrifugalizing the deposit and evaporating down the ether extract until it became of a sticky subsistence, then adding olive oil to a weighed amount to make up as far as possible a percentage strength.

The therapeutic use of this has been, as far as one can tell, rather more useful, though at present it is too early to compare the two methods clinically.

The Clinical Use of the Vaccine.—The method of treatment of voluntary cases at the Leper Asylum, Kemendine, by the use of a vaccine prepared from these cultures was commenced in March 1909.

Up to January 1910 the autoclaved vaccine prepared in the usual way was used: after that date the special washed bacillary vaccine, as described above, has been used. The ethereal extract of bacillary fat has been tried with beneficial result in a few cases, but it is at present too early to report on this variation.

Of the ten cases in which this treatment has been adopted, two have now recovered; two are so much improved that apparently the remnants of the disease are very slight; and the remaining six have all improved in a remarkable manner.

In these cases, with one exception, the injection of vaccine was the only treatment adopted.

A full clinical report on these cases will be published later on in one of the medical journals.

Part II.

THE CULTIVATION OF THE LEPROSY BACILLUS.

BY

CAPTAIN T. S. B. WILLIAMS, M.B., I.M.S.

During the last six years I have devoted special attention to the leprosy problem, and this paper contains a résumé of my results up to the present time. Prior to the beginning of 1909 my results were of an indefinite nature, and although useful to me, in helping me to judge the value of my later work, they would be out of place in this paper.

Up to the end of 1908 I had regarded the Bacillus of Leprosy as a true fission fungus, and had looked for it always as an acid-fast bacillus. My own and other people's negative results, combined at this time to cause in my mind a reconsideration of the problem. As a result of my work since the beginning of 1909, I have grown from five cases of leprosy, under circumstances which to my mind preclude contamination, two apparently different organisms. Two cases were in Persia and three in Bombay. Later work has tended to the belief that they are really the same organism, altered by environment. Shortly, I may say that in ordinary broth, I have grown a streptothrix, somewhat similar to that described by Professor Deycke, while in other special media, I have grown a "bacillus" very similar to that grown by Major Rost in Rangoon.

(A) BACTERIOLOGICAL RESULTS.

In all our experiments, it goes without saying, that every attempt has been made to avoid contamination, and ordinary media have always been used to control the technique employed, both at the initial transference of material and subsequently.

As stated above, I have grown two types of organisms: (*a*) a streptothrix, and (*b*) a "bacillus", which is almost certainly the bacillary form of a streptothrix. It will be more convenient to describe them separately.

(*a*)—*Streptothrix "leproides."*—This organism has been grown by me three times in Persia, from a Persian leper, and three times in Bombay, from a Goanese leper.

I.—In Persia.

The medium used was ordinary nutrient broth, which was inoculated from non-ulcerated lepromata of a Persian leper. The tubes were kept at room temperature. During the period of incubation the broth remained absolutely clear, and a very small deposit of the transferred material could be seen at the bottom of the tubes. After eight weeks' incubation small puff-ball growths appeared at the bottom of each tube, and gradually multiplied, growing upwards and adhering to the side of the tube.

On microscopical examination these growths were shown, after staining by Ziehl-Neilsen's method, to consist of a non-acid-fast streptothrix, with masses of acid-fast bacilli, lying amongst the meshes of the streptothrix. The first sub-culture showed the same non-acid-fast streptothrix, but the acid-fast bacilli were fewer in number, and from the third sub-culture onwards no acid-fast bacilli occur.

II.—In Bombay.

The same streptothrix, producing acid-fast nests has also been obtained three times from a Goanese leper. In these experiments the tubes were incubated at 37°C.

(i) In the first case six tubes of Potato broth were inoculated from different lepromata of said leper. Five tubes showed no growth. In one, however, at the end of ten days, there appeared a very small puff-ball growth in the bottom of the tube, the supernatant fluid remaining clear. Growth slowly increased, the colonies climbing up the side of the test-tube, to which they adhered.

Microscopically.—The growth was a non-acid fast streptothrix producing acid-fast nests. First sub-culture was similar, but acid-fast organisms were fewer and from third sub-culture no acid-fasts have been found.

(ii) *From a Rost's medium tube* of the "Bacillary" growth to be described in Section B, I inoculated a large flask of Potato broth. The Rost's medium tube contained acid-fast lepra bacilli, growing from the same Goanese leper. After ten days' incubation the flask of Potato broth showed one very small puff-ball. Growth increased very slowly. It was exactly similar to that described in the preceding paragraph. Microscopically, also, it was the same and its subsequent growth was similar.

(iii) *From a Rost's medium tube*, in which the "Bacillary" growth described in Section (*b*), had been growing for about a week, I made inoculations into six more tubes of Rost's medium. Nothing occurred in five tubes. In the sixth, after twenty-five days' incubation, four small puff-balls were observed

at the bottom of the tube. One removed for examination showed it to be similar microscopically to those described above. This growth, while similar microscopically to those grown in ordinary broth, and Potato broth, differed for a long time, in that it would only grow with the greatest difficulty. After much perseverance however it has gradually approximated culturally to the growth obtained in ordinary broth and Potato broth. While growing with great difficulty on agar media, it was of an orange-red tint. As its growth became more exuberant it has gradually lost this colouring, and now it shows the whitish yellow of the other growths, subcultured from the broth streptotriceæ.

III.—Growth characteristics of the Streptothrix leproides after initial culture.

(i) *Ordinary Broth.*—It grows readily from the second day as a flocculent membrane or puff-ball growth at the bottom of the tube. The medium remains clear.

(ii) *Ordinary Agar.*—It grows readily. There is not much surface growth. The organism digs its way into the agar medium, and the growth on the surface seems to get less and to be covered with a chalky powder.

(iii) *On Glycerine and Glucose Agar.*—It grows readily. Whereas in ordinary agar it digs into the medium everywhere, on Glycerine and Glucose agar it grows more luxuriantly as a definite membrane on the surface of the slope and the edges of the growth only grow into the medium.

(iv) *Maltose.*—Negative.

(v) *On unskimmed milk* at 37°C it grows, after about 10-14 days as a very definite coherent membrane on the surface of the cream. This membrane varies in colour according to the medium from which the sub-culture is made and owing also to variations in the medium, temperature, etc., etc., of which I am at present ignorant. The full-grown membrane, when subcultured from Potato broth, is a membrane very like that described by Deycke for his streptothrix, but the colour is more orange-yellow than orange-red. In the early part of its growth the membrane is all a non-acid-fast streptothrix. After about four weeks' growth some parts of it become very strongly acid-fast and after 6-8 weeks' growth it may break down into bacillary fragments, very similar morphologically to those obtained in Rost's medium and to be described in Section (*B*). These bacilli may be acid-fast or non-acid-fast. The streptothrix is distinctly gram-positive.

(*b*)—"*Bacillus*" *Lepræ.*—I have headed this "Bacillus" Lepræ to separate it distinctly from the more definite streptothrix growth. As stated

above, there are reasons for considering it as an altered form of the previously mentioned streptothrix.

For this series of experiments, I used either Major Rost's original medium, or an imitation prepared for me at Parel Laboratory. The medium prepared at Parel was as follows :—

(i) Lemco broth, without the addition of salt or peptone .	250 c.c.
(ii) Distilled water	250 c.c.
(iii) Milk	50 c.c.

The above differs from Major Rost's medium, in that the distilled water replaces an equal quantity of the Ngapee (rotten fish) distillate. On analysis by the Chemical Analyser to the Government of Bombay, it was declared to be "practically chloride-free."

This series of successful results numbers seventeen, divided between Bushire (in Persia) and Bombay, and I would emphasise that in each case where I have got the streptothrix growth, I have also in Rost's medium got the bacillary form of growth. Further, in two instances recorded above, the streptothrix form of growth with acid-fast nests of bacilli, has grown out of a tube of the bacillary form of growth. All these inoculations were made from non-ulcerated lepromata, and each experiment was controlled with ordinary media. No growth occurred in the controls of these seventeen experiments. After inoculation from the non-ulcerated lepromata all tubes were kept at 37°C. After two to three days it was quite evident that there was a multiplication of acid-fast bacilli going on. Positive results are only obtained by pipetting up from the lowest part of the tube a loopful from half way down the medium generally shows nothing.

After about seven days' incubation, a small flocculent membrane can be made out at the bottom of each tube. It is at first very small and is with difficulty distinguished from the sediment of the medium. This small membrane occurred in all cases. In two cases it was removed and preparations stained by Ziehl-Neilsen's method were prepared. These showed the membranes to consist of masses of acid-fast bacilli lying in a zoogloea substance. Further examination showed the basis of the growth to be a streptothrix varying in its acid-fast properties in different parts of the preparation. In this way growth proceeded very slowly, controls showing that there was nothing present capable of growing on the ordinary media. At the end of about two months' incubation, a low power field showed distinct masses of red, scattered about the field, which under the high power were seen to be made up of acid-fast bacilli.

Attempts were made frequently to obtain a growth on solid media, but for long without success. After about $2\frac{1}{2}$ months' incubation, in tubes of liquid

media which showed the best growth, it was noticed that, in addition to the acid-fast organisms, a non-acid-fast organism, similar in shape to the acid-fasts, began to appear. This non-acid-fast form is essentially a "diphtheroid" organism, and, whenever growth has taken a definite move forward, this non-acid-fast diphtheroid organism has appeared. This non-acid-fast form appeared too frequently in the liquid cultures for us to consider it as an accidental contamination, and when we succeeded in obtaining a growth on solid media we were confirmed in this opinion. After $2\frac{1}{2}$ months' incubation in Rost's medium from some of the strongest growths, we obtained with difficulty a growth on Lemco glucose agar (without the addition of NaCl or peptone). After 8-10 days' incubation on the agar, it was thought that there were signs of some growth; this was subcultured and a more definite growth was obtained. The growth was very weak and consisted of small separate dew drop colonies. Microscopically these consisted of the same acid-fast and non-acid-fast bacilli as were seen in the liquid culture.* By subsequent sub-culture in solid and liquid media the growth has been gradually strengthened until now we have obtained on solid media a strong growth of a yellow sticky character, which is similar to Major Rost's most typical growth in its strong acid-fastness; its pleomorphism, its yellow colour on solid media and in its power of producing a severe reaction in leper patients on whom it was tried. It differs in one important point—whereas Major Rost's growth is now dry and wrinkled, my growth is very sticky and greasy. This need not, however, be an essential difference, as Major Rost refers to the change of character of his growth after frequent sub-culture, and my own experience with one of his organisms is that of a sticky growth which may be changed by frequent culture into a dry, wrinkled form of growth.

On staining by the Ziehl-Neilsen method, one sees in a fairly typical culture of the original weak growth (i) acid-fast lepra bacilli, (ii) similar non-acid-fast dotted bacilli, which take the counterstain very faintly, and (iii) other "involution" forms, such as swollen bacilli, filaments with terminal clubs, coccoid forms, etc., which take the counterstain strongly.

With Gram's stain it is seen that the elements mentioned under (iii) are strongly gram-positive, while the others seem to vary in their staining reaction. Regarding the final strongly acid-fast growth it is more difficult to give a word picture of what is seen microscopically. What one sees depends on the

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* This weak growth has been continued up to the present time (30th January 1911) solely on agar and retains its original characteristics. The regularity with which the acid-fast bacilli recur in cultures and are found in single colonies disposes of the criticism made against the non-acid-fast streptothrix grown in broth, *viz.*, that the acid-fast nests of bacilli found in the broth growths were those originally transferred from the leper, which acid-fast bacilli had become accidentally entangled in the meshes of the contaminating streptothrix.

manner in which the streptothrix happens to be dividing at the moment. One may see acid-fast bacilli or acid-fast cocci, and in the groundwork one can make out at times parts of the non-acid fast streptothrix forms, which are the basis of the whole growth and in which the acid-fast forms develop.

(*c*) *Are the two organisms identical?*—Without being able to definitely settle the question by growing one into the other at will, we consider that we have good grounds for supposing them to be the same organism, altered by environment.

(i) The "Bacillus" lepræ has been grown from all five cases. The "bacillus" is almost certainly a phase of the life-history of a streptothrix, as evidenced by its growth in liquid media and by the forms seen in growth on solid media.

(ii) Both the streptothrix and the "Bacillus" were got comparatively frequently from two cases.

(iii) The streptothrix growth on milk breaks down into bacillary elements, both acid-fast and non-acid-fast, almost identical with those got by growing the "Bacillus" in liquid or on solid media.

(iv) The streptothrix has twice been grown from Rost's medium in which the bacillary form of the organism was growing.

(v) Deycke has grown a streptothrix which also breaks down into acid-fast bacilli.

(vi) Many observers have grown a non-acid-fast-diphtheroid organism. Both Major Rost's organism and mine are in their non-acid-fast stage, diphtheroid organisms. They are also both certainly streptotriceæ.

(vii) Both forms when made into a vaccine cause both general and local reaction in lepers. Controls on non-lepers point to the specific nature of the organisms.

(viii) Inoculation of both organisms into monkeys, rabbits, guinea-pigs and rats has been so far negative.

(ix) The streptothrix "leproides" when injected into lepers in its non-acid-fast form produces no reaction. It is only when a certain amount of acid-fastness has been developed that it produces a reaction.

(B) DISCUSSION OF RESULTS.

(*a*) Leprosy has been considered since the discovery of Hansen's bacillus a fission fungus disease, and indeed, it is so considered at the present time by most people.

To meet criticism of our work, based on this assumption, it will be well to clear the ground at once, and to point out that, although we find the lepra organism parasitically as an acid-fast bacillus, it is not therefore necessarily a fission fungus. It is, indeed, quite possible that it belongs to the mould fungi. It is impossible for me here to go into a general discussion of the Streptotriceæ and their varying forms. I would especially refer those interested to the Milroy lectures of 1910 by Foulerton. It will be seen from a study of these most interesting lectures that it is quite possible for a disease to be caused by a streptothrix, and yet the obvious parasitic form found may be always a bacillus, or some other broken down streptothrix form.

I would also refer to the following remarks of Unna as showing that he evidently has for long been considering possibilities apart from the acid-fast bacillus. In a paper printed in the "Transactions of the Bombay Medical Congress," 1909, he says, "as a matter of fact the leprosy organism includes a large and varied series of forms, which not only include the known bacilli and globi, but also others, different in morphology and staining reactions, and transitional forms, which are inseparable from the growth of the leprosy bacilli in the skin. I will not enter further into the peculiar morphological shapes, coccus-like granules and granular threads (coccothrix) which were found in my laboratory by Lutz (1885) and studied more closely by me and my pupil Spiegel, etc., etc." It is also interesting to note that Hansen himself in his early attempts to grow the organism, succeeded in getting a mycelial growth, which, however, later he admitted had nothing to do with the lepra organism. It is possible that with a knowledge of more recent work he may be inclined to change his opinion.

Again, is the leprosy bacillus always acid-fast ?

I quote from Allbutt and Rolleston's "System of Medicine" to show that it is, quite possibly, not always acid-fast. Dr. Abraham, the author of the article, says : "Herman has also shown that in young or recent nodules, similar masses of bacilli exist, which do not retain the red stain after immersion in weak acid, but are easily, subsequently, coloured with methylene blue. If, as he supposes, it is only the older (fuchsin stained) bacilli, that are resistant to the acid treatment, we may have some explanation of the general failure to recognise leprosy bacilli in the attempted cultivations, as will be shown further on."

And, lastly, I would like to draw attention to the fact that many workers, whose technique is beyond question, although they have failed to grow an acid-fast lepra bacillus, have described, all of them, the growth of a non-acid-fast diphtheroid bacillus. Amongst those who describe the growth of a non-

acid-fast diphtheroid bacillus are Babes, E. Levy, Czaplewsky, Spronk, Kedrowsky and Shiga.* Dean quotes Babes as having grown this non-acid-fast diphtheroid bacillus from twelve cases of leprosy, and a similar non-acid-fast diphtheroid bacillus has been grown from the leprosy-like disease occurring in rats. Babes evidently thought that his organism was very possibly the lepra bacillus. He said that although it was possibly the lepra organism in a non-acid-fast state, one could not consider it seriously unless—

(1) it was possible to grow it into the acid-fast state;

(2) it would produce a tuberculin-like reaction in lepers; or

(3) unless it could produce leprosy in animals.

The interest of this question of a "diphtheroid bacillus" and in Babes' remarks lies in the following facts:—

(1) Both Major Rost's and my organism are in their non-acid-fast states diphtheroid bacilli and certain growths tend to lose their acid-fastness and to become permanently non-acid-fast. With one of Major Rost's species, this organism definitely regains its acid-fastness on being grown in a fatty medium like milk.

(2) Both Major Rost's and my organism are capable of producing tuberculin-like reactions. Control injections on non-lepers point to the specific nature of the vaccines.

(3) Major Rost has in one monkey produced a nodular disease, similar to leprosy, and the nodule contained acid-fast bacilli.

(*b*) I have during the last two months been experimenting with a vaccine made from a two months' old streptothrix membrane grown on milk. This membrane is dissolved in olive oil. In the cases on whom it has been used it has produced, in appropriate doses, marked general reaction and also local reaction in the leprous lesions. These reactions have been followed by improvement in the leprous condition. Two anæsthetic cases treated with the vaccine have shown a rapid return of sensation in the affected areas.

A series of control experiments on non-lepers were carried out to exclude the olive oil alone, and then the vaccine. Five non-lepers were injected twice at weekly intervals with olive oil (equal in amount to the vaccine) and 5 non-lepers were injected twice with the vaccine. Those injected with olive oil were not affected in any way by the injection. Those injected with the

*It is particularly interesting to refer to Shiga's description (Transactions, Bombay Medical Congress, 1909) of the diphtheroid organism isolated by him. It will be seen that by inoculating fresh leprous tubercle on to potato glycerine agar with human serum, he obtained, after four weeks' incubation, the growth of a diphtheroid organism which was not acid-fast, but amongst the non-acid-fast organisms were seen acid-fast lepra-like bacilli. He thought, as did we, of the possibility of symbiosis, but further work showed that it was a pure culture. In his case, as in ours, the agglutination test was not certain, as leper serum and healthy serum reacted about the same.

vaccine suffered a certain amount at the site of injection for about 24 hour and two had slight fever. None of the controls, however, in any way approached the severe reaction which I have obtained in cases of leprosy. In the anæsthetic cases, where one would expect the numbers of bacilli to be less than in pronounced tubercular cases, reaction has been very slight,—hardly more than in the controls. In definite tubercular cases reaction has been severe, and judging by my results on five cases—three tubercular and two anæsthetic,—the reaction appears to be proportionate to the severity of the disease.*

(*c*) In a subject like leprosy, it is from every point of view necessary to go slowly and not to make definite claims until they can be proved up to the hilt. I am well aware that our present work does not reach this standard. I think, however, that the results merit favourable consideration, and they are put forward at this juncture in the hope that others interested in leprosy may be induced to attack the subject along the same lines.

---

* Since writing the above in September 1910, we have discarded the vaccine made by dissolving the streptothrix membrane in olive oil.

For some time we experimented with a vaccine made by scraping the acid-fast growth off agar tubes; drying in vacuo; powdering in a mortar; and then dissolving the material in normal salt so ution. This gave rise to similar reactions, both general and local, to those produced by the olive oil vaccine. The results were not, however, regular enough to be satisfactory. After consultation with Major Rost, we decided to try the injection of a six weeks' old bouillon culture of the organism. These experiments have only been in operation for the last three weeks and one would not be justified in saying much about them, but there can be no doubt that the bouillon vaccine is much more potent in so far as producing a reaction is concerned. The definite character, moreover, of the local reaction in the leprous lesions, in many cases, supports the view that we are dealing with a specific remedy (T. S. B. W., 30-1-11.)

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BY

MAJOR E. R. ROST, I.M.S.

PART II.

The Cultivation of the Leprosy Bacillus

BY

CAPTAIN T. S. B. WILLIAMS, M.B., I.M.S.
(EDITED BY THE DIRECTOR-GENERAL, INDIAN MEDICAL SERVICE.)

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